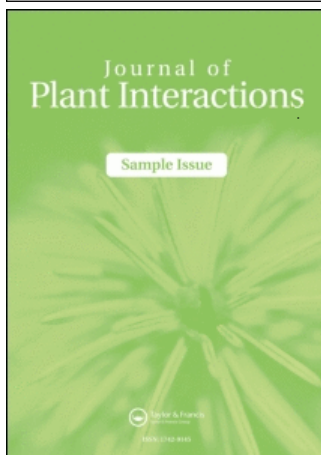


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Challenges, achievements and opportunities in allelopathy research

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REVIEW

Challenges, achievements and opportunities in allelopathy research

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Abstract

Allelopathy is defined as the suppression of any aspect of growth and/or development of one plant by another through the release of chemical compounds. Although allelopathic interference has been demonstrated many times using *in vitro* experiments, few studies have clearly demonstrated allelopathy in natural settings. This difficulty reflects the complexity in examining and demonstrating allelopathic interactions under field conditions. In this paper we address a number of issues related to the complexity of allelopathic interference in higher plants: These are: (i) is a demonstrated pattern or zone of inhibition important in documenting allelopathy? (ii) is it ecologically relevant to explain the allelopathic potential of a species based on a single bioactive chemical? (iii) what is the significance of the various modes of allelochemical release from the plant into the environment? (iv) do soil characteristics clearly influence allelopathic activity? (v) is it necessary to exclude other plant interference mechanisms?, and (vi) how can new achievements in allelopathy research aid in solving problems related to relevant ecological issues encountered in research conducted upon natural systems and agroecosystems? A greater knowledge of plant interactions in ecologically relevant environments, as well as the study of biochemical pathways, will enhance our understanding of the role of allelopathy in agricultural and natural settings. In addition, novel findings related to the relevant enzymes and genes involved in production of putative allelochemicals, allelochemical persistence in the rhizosphere, the molecular target sites of allelochemicals in sensitive plant species and the influence of allelochemicals upon other organisms will likely lead to enhanced utilization of natural products for pest management or as pharmaceuticals and nutraceuticals. This review will address these recent findings, as well as the major challenges which continue to influence the outcomes of allelopathy research.

Keywords: Allelopathy, allelochemicals, competition, microorganisms, interference, natural products, soil

Introduction

The phenomenon of growth inhibition of one plant through the release of chemicals from another plant into the environment is generally defined as allelopathy (Inderjit & Callaway 2003). The term 'allelopathy' was first utilized by Hans Molisch (1937) from a physiological perspective to describe the effect of ethylene on fruit ripening. The identification of allelopathy as the potential cause of vegetation patterning however, proved difficult due to the dynamic nature of soil and complex ecosystem interactions. Muller et al. (1964) first reported that zones of inhibition around shrubs such as *Salvia leucophylla*, *Artemisia californica* and *Adenostoma fasciculatum* were likely due to volatile chemicals released by these shrubs. Bartholomew (1970), however, ruled out the possibility of allelopathy as a potential cause for these zones by creating barriers restricting mammalian movement. He

eventually showed that grazing, trampling or seed feeding activities of animals resulted in bare concentric areas surrounding *Salvia* shrubs. This finding, which discounted the highly cited study of Muller et al. (1964), proved a major setback to allelopathy research and resulted in considerable criticism of allelopathy by certain prominent ecologists. John Harper (1977) was one ecologist who reported that, "Almost all species can, by appropriate digestion, extraction and concentration, be persuaded to yield a product that is toxic to one species or another." While evaluating the work of Elroy Rice, a pioneer in allelopathy research on old-field succession, he commented, "This series of experiments gave a suggestive, not conclusive, evidence of a toxic concentration in specialized laboratory conditions, and they provide no evidence in support of allelopathy being operative in field conditions."

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Wardle et al. (1998) summarize the problems associated with recognition of allelopathy as a valid field of research by stating that, "...acceptance (of allelopathy) by plant ecologists has been limited because of methodological problems as well as difficulties in relating results of bioassays used for testing allelopathy to vegetation patterning in the field." One reason for this could be that allelopathy was largely observed in past studies at the population level. Furthermore, allelochemicals may influence ecological patterning through their direct release, their degraded or transformed products, their effects on physical, chemical and biological soil characteristics, and/or by inducing the release of biologically active compounds by a third species (Inderjit & Keating 1999). Two problems related to both field and laboratory research conducted on allelopathy – (1) the utilization of inadequate methodology with regards to chemical extraction and subsequent identification, and (2) the lack of clear evidence of allelopathy in field settings contribute to the inability of researchers to address key points raised in the ecological literature.

While elaborating criteria that could be utilized to further demonstrate the occurrence of allelopathy, Willis (1985) stressed the importance of: (i) a pattern of inhibition of one species or plant by another, (ii) phytotoxin production by the aggressive plant, (iii) knowledge of the mode of toxin release from the plant into the environment, (iv) toxin transport and/or accumulation in the environment (v) toxin uptake, and (vi) exclusion of physical or biotic factors influencing plant interference. Blum et al. (1999), however, state, "Because no field observations or controlled field experiments have ever met the criteria elaborated by Willis (1985) in their entirety, the idealized concept of the allelopathic phenomenon has never actually been confirmed in the field." Currently, we believe that there is no pressing need to further elaborate upon the different criteria to demonstrate the occurrence of allelopathy. The purpose of most scientific studies is to gain an understanding of the phenomenon in question to the point of being able to make reasonable predictions about its existence. We propose that focus should be on 'testing a prediction' rather than 'demonstrating a phenomenon.' In our opinion, most of the criteria published, which suggest how one might conclusively demonstrate allelopathy, are unattainable and in some cases, artificial. Perhaps more importantly, one must identify the mechanism(s) involved in allelopathic interactions, particularly in natural settings. The objectives of this review are to: (i) address certain critical questions that pose challenges to current researchers working in this field, and (ii) highlight some recent achievements in the area of allelopathy.

How essential are inhibition zones in demonstrating allelopathy?

The discovery of bare rings, inhibition zones, monocultures and root segregation may establish an appropriate ecological context for further investigation of allelopathic interference. However, alternate explanations for vegetation patterning are certainly possible (Inderjit & Weiner 2001). Many studies have shown inhibition zones around putative aggressors, and attempted correlation of the existence of bare rings with allelopathic activity (Muller et al. 1964, Katz et al. 1987, Weidenhamer & Romeo 1989). As described above, bare zones may or may not be due to the presence of bioactive allelochemicals and should not be taken as sole evidence to initiate the investigation of allelopathic interference. In agroecosystems, where incorporation of plant residue is common, such inhibition zones do not generally exist, but research has clearly demonstrated the existence of potent allelopathic activities (Hamdi et al. 2001, Inderjit et al. 2004). Mahall and Callaway (1992a, b) investigated underground interactions among and between two Mojave-desert shrubs *Ambrosia dumosa* and *Larrea tridentata*. They found that roots of *A. dumosa* clearly "avoided" or grew away from the roots of other *A. dumosa* plants; however, roots of *L. tridentata* inhibited root growth of both *A. dumosa* and *L. tridentata*. Later, root-mediated allelopathy was implicated in explaining *L. tridentata* interference to *A. dumosa* (Mahall & Callaway 1992b). Inhibition zones located above the soil surface, around established vegetation, or lack thereof, can therefore be misleading. By utilization of spatially segregated root system studies, clear evidence for allelopathic interference below ground, in the absence of above ground zones of inhibition has been obtained (Schenck et al. 1999, Inderjit & Callaway 2003). Observance of a clear zone of inhibition is therefore not essential to demonstrate the occurrence of allelopathy.

How ecologically relevant it is to attribute the allelopathic potential of a species to a single bioactive compound?

In most cases, allelopathic activity is associated with mixtures of several compounds rather than with individual compounds (Einhellig 1995). Artemisinin, a potent phytotoxin from the annual wormwood (*Artemisia annua*), is reported to inhibit the growth of *Amaranthus retroflexus*, *Ipomoea lacunosa*, *A. annua* and *Portulaca oleracea* (Duke et al. 1987). Soil amended with pure artemisinin was less inhibitory to the growth of *A. retroflexus* than soil amended with the more complex mixture of *A. annua* leaf extract (Lydon et al. 1997). As is often the case, all of the phytotoxic activity of a plant could not be attributed to a single compound.

Joint action of chemicals in a mixture is potentially important in explaining allelopathic interference in natural settings. In fact, the concentration of each chemical in a mixture might be significantly less than the concentration of an individual chemical required to induce an inhibitory effect on higher plant growth (Blum 1996). In pharmaceutical and herbicide research, the Additive Dose Model (ADM) and the Multiplicative Survival Model (MSM) have often been employed to study the joint action of chemicals in a mixture. While the ADM assumes the chemicals to have similar molecular targets in the receiver plant, the MSM assumes that chemicals have different molecular targets and exert their effects independently of each other in the receiver plant (Morse 1978). The ADM assumes that chemicals in a mixture can replace each other on the basis of their biological exchange rate or their "relative potency", and any departure of the effect of mixtures from the ADM is characterized by either reduced (antagonistic) or enhanced (synergistic) effects (Streibig & Jensen 2000). When the ADM and MSM are compared at low and high effect levels of a mixture, the relationship between the two models depend upon the slopes of the curves and mixture dose administered (Drescher & Boedeker 1995). Interaction of two compounds with their absorption, uptake, translocation, or binding at site(s) of action may result in deviation from the ADM.

In allelopathy research, equimolar concentrations of chemicals are used to prepare mixtures, and the ED_{50} or ED_{90} values of the compounds are not generally considered (Einhellig 1989). Inderjit et al. (2002) studied the joint action of three phenolic acids, *p*-hydroxybenzoic, *p*-coumaric and ferulic acids, in binary mixtures. On the basis of the distribution of the mixtures along the ED_{50} isobole, these authors found that the majority of mixtures are above the ADM isoboles at the response levels of ED_{50} , and thus exert antagonism relative to the ADM. Therefore, one appropriate way to describe the joint action of allelochemicals is to use the response curves of allelochemicals applied alone and in mixtures, and incorporate various joint action reference models to fully explain the results observed, e.g., the additive dose model and the multiplicative survival model (Streibig 1992). Allelochemicals with similar modes of action are often used in experiments to examine their joint action in a mixture. Root exudates or foliar leachates, however, may also contain diverse phytotoxins with different modes of action. Large differences in their relative potencies and the activity of the natural mixture complicate the study. Inderjit et al. (2005) studied experimental difficulties in the evaluation of comparative phytotoxicity of chemicals with different molecular targets. The authors focused upon three chemicals: benzoic acid, a natural product, and the two synthetic herbicides, isoxaflutole and rimsulfuron.

Benzoic acid either inhibits nutrient uptake or causes membrane-associated disturbances (Einhellig 1995, Inderjit et al. 2002). Isoxaflutole and rimsulfuron inhibit the enzymes HPPD (hydroxyphenylpyruvate dioxygenase) and ALS (acetolactate synthase), respectively (Fedtke & Duke 2005). Not surprisingly, a large difference in the bioactivity of benzoic acid ($\approx 600\text{--}2500$ mg/l) and rimsulfuron or isoxaflutole ($\approx 0.01\text{--}0.5$ mg/l) was observed. Due to these large differences in ED_{50} values, it is particularly difficult to prepare binary mixtures to further study their joint action. Another problem encountered is the different response of bioassay species to individual chemicals. While the phytotoxic activity of benzoic acid and rimsulfuron can be examined using a 7-d duration Petri dish experiment, isoxaflutole did not show any activity. Both isoxaflutole and rimsulfuron showed activity in pot experiments conducted over a one-month period, but benzoic acid had no persistent activity over the same time frame. One problem in selecting a common bioassay design for mixtures of allelochemicals is the potential difference in soil persistence of the components in the mixture. Another problem encountered is the large number of identified secondary products in certain mixtures of allelochemicals. For example, the volatile oils collected from mugwort (*Artemisia vulgaris*) foliage contain over 15 major components (Barney et al. 2005a). Investigating allelopathic potential of a species by examining only a single compound instead of a complex mixture may not have much relevance in terms of explaining field patterning in ecosystems. There is a critical need to examine lessons learned from herbicide research to enhance the understanding of allelopathy from the perspective of the joint action of chemicals in a mixture.

During phytochemical analysis, attention is often paid only to biologically active compounds with "appropriate" specific activities occurring in proportionately large concentrations. Compounds present in relatively low concentrations often remain neglected. Under these conditions, it is difficult to gain information about the biological activity of the minor components of the mixture, or impact of alterations in composition of the mixture upon plant growth. Consideration of both total activity and specific activity of allelochemicals in the soil is needed to understand the potential for allelopathic effects (Hiradate 2006). Furthermore, bioassay-guided fractionation is not always the best approach to utilize in studies of the specific activity of allelochemicals, given that the allelochemicals present in the plant may be rapidly converted into more active constituents once they arrive in the rhizosphere. For example, a more phytotoxic compound, APO (2-aminophenoxazine-one), is generated from the graminaceous allelochemical DI-BOA (4-hydroxy-(2H)-1,4-benzoxazin-3(4H)-one)

by microbial alteration in soil (Macias et al. 2005a,b). It is therefore equally important to evaluate the concentration and the form an allelochemical is available to the target species, and to consider how the qualitative effects and quantitative concentration of a given compound are influenced by habitat, by physical, chemical and biological soil factors, by climatic conditions, and by other parameters. Enhanced ability to extract and detect allelochemicals in soil solution will aid in our ability to monitor the dynamics of allelochemical flux in field conditions.

What is the importance of the mode of allelochemical release from the plant into the environment, and how do we refine our studies in this area?

Phytotoxic chemicals from plants can enter the environment either through volatilization, foliar leaching, root exudation, residue decomposition or through leaching from plant litter (Birkett et al. 2001). Root exudates are important in terms of contributing chemicals directly into the rhizosphere environment (Inderjit & Weston 2003), but potential leaching of chemicals from aerial plant tissues should not be neglected. (–)-Catechin, a component of the root exudates of spotted knapweed (*Centaurea maculosa*), was reported to be the key allelochemical associated with the invasive success of spotted knapweed (Walker et al. 2003, Bais et al. 2002, 2003), although recent studies question the activity and availability of catechin as a phytoinhibitor in the soil rhizosphere (Blair et al. 2005). Although there are reports on the presence of cnicin, a relatively active sesquiterpene lactone in the foliage of spotted knapweed (Kelsey & Locken 1987, Locken & Kelsey 1987), its role in the invasive success of spotted knapweed has not been documented. One reason cnicin may not have been investigated further with regards to spotted knapweed invasion could be the fact that its presence is currently associated only with knapweed foliage. Further studies addressing allelochemical production and release by *Centaurea* spp. in both *in vitro* and field settings, where allelochemical content and activity are carefully monitored over time, may lead to more conclusive findings related to the biological activity of catechins and potentially other unidentified allelochemicals.

Precipitation or wind movement may result in leaching of foliage and associated allelochemicals into the surrounding soil. For example, catmint (*Nepeta × faassenii*) is an ornamental groundcover which possesses numerous secretory glands on the abaxial surfaces of its leaves. The volatile mixture released by this plant over time contains numerous secondary products, three of which are related nepetalactones occurring in high concentrations. The volatiles are potent inhibitors of seedling growth and germination in enclosed bioassays (Eom et al. 2006). Barney et al. (2005a) recently showed that

mugwort (*Artemisia vulgaris*) also produces a cocktail of volatiles which can bind to soil particles in enclosed soil bioassays and interfere with plant growth and germination. Foliar tissues of many plants disperse large quantities of volatile mixtures which can be noted in and around established stands of certain perennials on warm, sunny days (Barney and Eom, personal communication). In addition, in three years of field experimentation, weed growth under established catmint and mugwort plantings was negligible, compared to other groundcovers or mixed vegetation (Barney et al. 2005b; Eom et al. 2006). Recent field experiments have suggested that volatiles released by mugwort may be associated with inhibition of neighboring plant growth as indicated by necrosis of young tissue (Barney 2006).

By improving our ability to collect and detect trace quantities of allelochemicals in mixtures on the plant foliar surface in the form of oils, associated with trichomes, glands or waxes, or the collection of minute quantities of root exudates from living roots coupled to the use of GC or HPLC with mass spectrometry, we have been able to characterize the site(s) of production of allelochemicals in plant structures and organelles, and gained valuable information about their potential release over time. The use of microscopy, including scanning and transmission electron microscopy, has also aided the study of root exudation and glandular release of allelochemicals (Duke & Paul 1993, Duke et al. 1994, Bertin et al. 2003, Czarnota et al. 2003a, Eom et al. 2006). By localizing production of allelochemicals, one can determine if genes and enzymes for biochemical production are specific to certain cells, tissues, or organs and how and when allelochemicals are released over time with respect to plant growth and development.

Quantity and quality of allelochemical production can vary significantly during plant development. If allelochemical production is enhanced in the early stages of plant development, such as is the case with the production of dhurrin or other related phenolics by *Sorghum* spp. (Weston et al. 1989), one can speculate that these chemicals may play an important defensive role in situations where the immature plant is undergoing establishment in a challenging environment. If enhanced allelochemical production is associated with increasing plant maturity, then the defensive role of allelochemicals may be considerably more important in promoting the ability of higher plants to successfully reproduce and disseminate seeds or propagules. Relatively few, if any, studies have been conducted to closely examine the ability of plants to produce and disseminate allelochemicals through living plant parts or residues throughout their lifecycle. Those studies that have been performed have generally suggested that changes in the production of multiple compounds or allelochemicals occur during plant development and in

response to environmental and biotic stimuli, sometimes in both roots and foliage (Weston & Duke 2003). This further complicates the study of allelochemical release, but extensive knowledge of the site of production of allelochemicals will allow the design of more appropriate assays to quantify production and release over time.

How much do soil characteristics influence allelopathic activity?

Studying the allelopathic potential of chemicals after their entry into soil environment is a major challenge encountered in allelopathy research. All plants synthesize a myriad of chemicals, many of which may be phytotoxic in bioassays without soil; nevertheless, these chemicals may not be involved in allelopathic suppression of a plant species. The fate of many allelochemicals in the soil environment is relatively unknown. After their release, allelochemicals are exposed to physical (e.g. sorption), chemical (e.g., direct oxidation or oxidation by metal ions) and biological (e.g. microbial degradation) soil factors (Huang et al. 1999, Inderjit 2001, Inderjit & Bhowmik 2004, Kaur et al. 2005). There is a need to generate data on the residence time, degradation and transformation of allelochemicals in soil, and their qualitative and quantitative differences in the rhizosphere versus bulk soil.

One problem encountered in the assessment of allelochemical dynamics in soil settings is that these chemicals may be present in minute quantities. There are many sinks present in the soil that determine the fate of allelochemicals. Depending on the extraction method, quantification of a compound in soil may only be a reflection of the quantities that remain after one or more of these potential sinks have been more or less saturated. Phytotoxicity is largely a function of the concentration of bioavailable allelochemicals remaining in the soil environment, but it is particularly difficult to precisely determine the bioavailable and non-bioavailable fractions of allelochemicals and other xenobiotics in the soil environment. Plants can develop root systems possessing enormous length and great surface area, and this is increased in the case of mycorrhizal roots. Roots of competing plants may come in direct contact with one another. In such situations, it is difficult to determine if transfer of allelochemicals is soil-mediated or how much is directly transferred through actual root-root contact. Research with black walnut (*Juglans nigra*), which releases the allelochemical juglone, has shown that large quantities are released by living roots directly into the rhizosphere surrounding living fibrous roots. While studying spatio-temporal variation in soil, juglone concentrations in black walnut-maize (*Zea mays*) alley cropping system, Jose and Gillespie (1998) found a decline in the juglone levels in soil with increasing distance from the tree. However, the

seasonal dynamics of juglone content varied with distance from the tree. Juglone concentration within a tree row was highest in fall, but at a distance of 0.9 m from the tree row, it was highest during spring and summer. Juglone degrades overtime, but can remain active in the soil solution, depending upon environmental conditions. Proximity to living walnut roots apparently determines the extent of phytotoxicity. Accumulation of juglone at phytotoxic levels also depends upon the microbial ecology of soils. The bacterium *Pseudomonas putida*, isolated from soils beneath walnut in Germany, was shown to convert juglone to 2-hydroxymuconic acid (Rettenmaier et al. 1983). Soil ecology is therefore an understudied but important determinant of allelopathic activities (Inderjit 2001). As Inderjit and Weiner (2001) stated, "Putting allelopathy in the context of soil ecology can further allelopathy research and reduce some of the less fruitful controversy surrounding the phenomenon."

The best soil-applied, synthetic herbicides, such as trifluralin [2,6-dinitro-N,N-dipropyl-4-(trifluoromethyl)benzenamine], are highly lipophilic. Such compounds adhere to soil particles and do not readily leach out of the root zone of target weeds. Even though the concentration in soil water is very low, the herbicide flux from soil particles to soil water to lipophilic domains, such as cell membranes of roots, can be rapid, resulting in accumulation of the compounds to phytotoxic levels. Recent work by Weidenhamer (2005), using fibers and tubes treated with a lipophilic material that takes up lipophilic material from soil solutions, indicates that lipophilic allelochemicals such as sorgoleone function in much the same way as soil-applied, synthetic herbicides. Understanding the dynamics of allelochemicals in the rhizosphere is crucial in understanding allelopathic mechanisms and assessing the importance of allelopathic processes in plant communities.

In another example, *Chenopodium murale*, a weedy native of Europe, has invaded cultivated fields, roadsides, abandoned soils and wastelands in Northern India. Leaves of *C. murale* are incorporated into the soil during tillage, and water-soluble substances are released after fields are flooded prior planting of lowland rice. El-Khatib et al. (2003) reported the allelopathic activities of *C. murale* rhizosphere soil and soil amended with its leaves. Inderjit (2006) found that activated carbon could not completely eliminate the inhibitory effects of soil amended with high amounts of *C. murale* leachate, but phytotoxic effects were largely eliminated after addition of N-fertilizers. Phytotoxicity of *C. murale* leaf leachate could be associated with immobilization of inorganic nitrogen due to microbial activity, a response that could be misinterpreted as an allelopathic effect. Soil properties and edaphic effects likely play important roles in influencing the extent of allelopathic

interactions over time. To further evaluate the ecological significance of secondary products in plant interactions, there is a need to determine systematically whether allelochemical effects (i) are temporal or more persistent, (ii) exist at the species and/or community level, and (iii) if an allelochemical release/degradation/sink influx-outflux equilibrium is ever achieved over time in the rhizosphere. Focused collaborations with soil chemists and soil microbiologists will be needed to further advance the study of allelochemicals in the soil rhizosphere.

How imperative it is to separate allelopathy from other interference mechanisms?

Although C. H. Muller and E. L. Rice attempted to evaluate the cause for patterning of plant species in natural settings over time, cause and effect cannot be established by the process of exclusion alone. Numerous factors, including resources, pest incidence, and presence of foraging birds, mollusks, and mammals, must be considered in order to evaluate or establish the mechanism for species patterning (Harper 1977). Several mechanisms of interference including resource competition, allelopathy, microbial nutrient immobilization, or nutrient interference often operate in parallel (Inderjit & Del Moral 1997), and allelochemical interference can be also influenced by various abiotic and biotic stress factors. Separating allelopathy from resource competition is often considered critical to establish allelopathy as a probable cause of interference (Nilsson 1994, Weidenhamer 1996). But as Zimdahl (1993, p 110) states, "It is not wrong to separate the elements of competition experimentally, but it is wrong to assume that plants do so, and it is nearly impossible to separate them in nature." We agree with Zimdahl's statement, and although controlled experiments can demonstrate allelopathy and separate it from other mechanisms of interference (e.g., resource competition), separation is not likely to occur in natural settings. In fact, it may be more relevant to predict the level of contribution that competition and allelopathy contribute to plant interference in an interaction study (Hoffman et al. 1996). Although we have limited data on this subject in natural settings, studies have been performed under controlled greenhouse conditions attempting to predict or quantify the interference observed due to allelopathy and/or competition for resources (Hoffman et al. 1996). Mahall and Callaway (1992b) determined that the interactions among roots of two Mojave-desert shrubs, *Ambrosia dumosa* and *Larrea tridentata* could not be explained only by competition for limiting resources. They found that inhibitory substances released from the roots of *L. tridentata* play an important role in the suppression of neighboring roots. As suggested by these authors, *A. dumosa* may competitively remove water otherwise available to *L. tridentata*. Roots of *L. tridentata*

may also inhibit roots of *A. dumosa* by releasing inhibitory substances. This is an example where both resource competition and allelopathy are likely to operate in parallel. The possibility of allelopathy as a probable cause of plant growth inhibition in some natural systems is not denied, but it has not yet been proven to be the sole factor of interference in any study. From a mechanistic standpoint, evidence for any biological/ecological phenomenon is often 'circumstantial', and resource competition is no exception. However, we can surmise that resource competition usually exists continuously, which may be seldom the case for allelopathy.

Allelopathy is more likely to play a key role in agro-ecosystems. Species have not coevolved over extended periods of time in most agro-ecosystems, so that it is less likely that there is resistance of the target species to an allelochemical. Weston (1996) discussed the allelopathic suppression of weeds by various cover crops, including buckwheat (*Fagopyron sagittatum*), black mustard (*Brassica nigra*), sorghum (*Sorghum bicolor*), wheat (*Triticum aestivum*) and rye (*Secale cereale*). The cereal crops, such as wheat, maize and rye have strong potential to produce hydroxamic acids and other phenolics which are released into soils through root exudation (Niemeyer & Perez 1995). Fujii et al. (1992) found that velvetbean (*Mucuna pruriens* var. *utilis*) has a beneficial impact on yield of graminaceous crops, and has the ability to smother noxious weeds, such as purple nutsedge (*Cyperus rotundus*) and cogongrass (*Imperata cylindrica*). These authors found l-DOPA (L-3,4-dihydroxyphenylalanine) as the main compound responsible for velvetbean allelopathic activity. In many agro-ecosystems, unharvested stubble or straw of rice (*Oryza sativa*) and wheat get incorporated in soil. Following irrigation, water-soluble chemicals may rapidly impact weeds in the next crop. Hamdi et al. (2001) reported that allelopathy is one cause of phytotoxicity of soil-incorporated wheat straw to perennial ryegrass (*Lolium perenne*). Using experimental modifications of the soil (such as activated carbon, soil sterilization, N amendments), Inderjit et al. (2004) found that water-soluble compounds released from rice straw were among the main factors causing inhibition of mustard (*Brassica napus* var. *toria*) seedling growth.

Williamson (1990) stated that a common problem encountered in allelochemical research is related to the absence of an appropriate methodology that can separate the effects of allelopathic compounds from other factors. Recent authors have suggested that the use of postulates similar to those described by Koch in identifying causal organisms of disease are generally not appropriate for assessing allelopathic interference because (i) allelochemicals are prone to chemical oxidation and microbial degradation in the environment, (ii) allelochemicals do not reproduce or generally proliferate over time, and (iii)

allelopathic activity is often due to the joint action of chemicals in a mixture rather than to one specific chemical. Williamson proposed that studies should be designed to eliminate all factors but the allelochemicals in question. This approach, although widely utilized, presents certain problems. For example, root exudates or foliar leachates are mixtures of organic constituents including sugars, enzymes, amino acids, and even inorganics. Growth assays performed with exudates or leachates containing complex mixtures cannot generally be simulated by utilizing single chemicals or simple mixtures of chemicals. Many of the components of root exudates and/or foliar leachates in natural systems influence both organic and inorganic components of the soil (Inderjit & Dakshini 1994a, 1994b). Growth responses due to allelochemicals could be due to their direct effects, the direct effects of their degradation byproducts or to the influence of some or all of these compounds on soil edaphic factors (Inderjit & Weiner 2001). Generally, chemicals present in very low concentrations are not considered as crucial in interfering with plant growth as those present in high concentrations. However, the relative phytotoxicity of compounds can vary by orders of magnitude, and, thus specific activities of compounds are important in assessing contributions to allelopathy (Hiradate 2006). Allelopathic activities may also be due to the joint action of allelochemicals and to their interaction with other organic compounds present in the soil substratum (Blum 1996, Inderjit et al. 2002).

Pluchea lanceolata (Asteraceae) is a perennial weed encountered in the semiarid regions of India. It is an aggressive perennial weed with a deep subterranean root system which acts as a strong competitor in nutrient-rich environments (cultivated fields) and is stress tolerant in infertile soils. The presence of phenolic compounds in the foliar leachates and root exudates of *Pluchea* and in surrounding soil indicates the species potential to interfere successfully with other plants by allelopathy (Inderjit & Dakshini 1994a, Inderjit 1998). When compared to *Pluchea*-free soils, *Pluchea*-infested soil had higher levels of total phenolic compounds and lower soil electrical conductivity and soluble chloride (Inderjit 1998). Some Indian farmers cultivate fields twice a year for two crops, while others produce only one crop. Greater soil disturbance (twice-a-year plowing *versus* once-a-year plowing) resulted in greater release of phenolics and a lower amount of exchangeable calcium in infested soils (Inderjit & Dakshini 1996). Under nutrient stress (infertile vs. fertile soil), the concentration of total phenolics and calcium increased (Inderjit & Dakshini 1994c). Qualitative and quantitative variation in phenolics and qualitative differences in soil fungal populations were also observed in *Pluchea*-infested soils sampled from different geographical locations (Inderjit et al. 1996). This example clearly illustrates that soil biota,

soil nutrients, site variation, habitat specificity and agricultural practices all play important roles in *Pluchea* interference to crop plants, in addition to potential allelochemicals released by *Pluchea* into the soil rhizosphere.

In the absence of other ecological processes, the phenomenon of allelopathic interference cannot solely explain *Pluchea* fitness and competitiveness. Plants may dominate an ecosystem for many different reasons (Dakshini & Sabina 1981, Inderjit & Del Moral 1997, Inderjit & Weiner 2001). The relative importance of allelopathy in *Pluchea* interference may vary depending upon the environmental factors, including soil characteristics, climate and stress. When investigating the plausible role of a particular ecological factor in plant fitness, clearly defining the ecological conditions encountered in each system is critical. Species strategies may vary between two different sites. For example, soil biota (Callaway et al. 2004) and resulting release of allelochemicals in native and invaded land (Callaway & Aschehoug 2000, Vivanco et al. 2004), allelochemicals in disturbed and relatively less disturbed soil (Inderjit & Dakshini 1996), and allelochemicals in nutrient-rich and nutrient-poor soil (Inderjit & Dakshini 1994c) all vary considerably. When attempting to thoroughly describe a particular mechanism associated with plant fitness, it is important to evaluate the environment or ecological factors under which the system is operating. Although allelochemicals influence community structure and ecosystem processes, allelopathy is now generally investigated using population-level approaches. An ecosystem-level approach which involves close examination of a variety of organisms or key players co-existing in the system and specific impacts of allelochemicals upon their performance and survival should lead to a better understanding of allelochemical influence on plant interference and community structure (Wardle et al. 1998).

How can new frontiers in allelopathy research help in solving problems and answering question in agroecosystems and natural systems?

Despite the difficulties in studying and clearly evaluating allelopathic interference in a natural setting, some progress is being made in terms of generating strong circumstantial evidence in support of the existence of allelopathy and also in understanding the genetic, biochemical, and physiological aspects of production and effects of plant allelochemicals under more controlled conditions (see Bais et al. 2003, Inderjit & Callaway 2003, Inderjit & Duke 2003, Weston & Duke 2003, Baerson et al. 2005a). From a long-term ecological perspective, Preston and Baldwin (1999) investigated allelopathic interactions of sagebrush (*Artemisia tridentata* ssp. *tridentata*) with seed banks of *Nicotiana attenuata* through the release of methyl jasmonate (MeJA), a

highly active volatile compound. Inhibition of tobacco germination was found to potentially benefit sagebrush by reducing direct interference with tobacco until competition was later reduced by the action of fire (see also Preston et al. 2002).

Callaway and his colleagues (Callaway & Aschehoug 2000, Bais et al. 2003, Callaway & Ridenour 2004, Vivanco et al. 2004) tested the hypothesis that allelopathy may be a potential cause for the invasive success of noxious non-native weeds such as spotted and diffuse knapweed in North America. *Centaurea maculosa* and *C. diffusa* are reported to release (–)-catechin or 8-hydroxyquinoline, respectively, in both native and naturalized ranges. The novel weapons hypothesis (NWH) identified allelopathy as one of the mechanisms to potentially explain the invasive success of *Centaurea* spp. in North America (Callaway & Aschehoug 2000, Vivanco et al. 2004). It suggests that allelochemicals provide greater competitive advantage to an invader in its naturalized range than in its native land, because natural neighbors with which it has co-evolved have evolved resistance to the allelochemicals (Callaway & Ridenour 2004). In our opinion, the term NWH is not an ecologically “appropriate” term because these compounds are clearly not novel in terms of their chemistry, distribution or activity.

Chemicals, such as (–)-catechin or 8-hydroxyquinoline, are commonly encountered in both native European soils of *Centaurea* spp. and invaded North American soils, but the concentrations of these allelochemicals are reported to be higher in North American soils (Bais et al. 2002, Weir et al. 2003), although the amounts found have been variable (Blair et al. 2005, Perry et al. 2005). These compounds are not novel from a structural standpoint as they have been previously reported as occurring in other plants. In fact, these chemicals may be entering novel environments, and, thus, this novel environmental interaction (the soil setting) may account for enhanced phytotoxicity. Whether any native species are biosynthesizing the same compounds has not been fully explored. Given recent research findings involving knapweed, allelochemicals are likely to be present in both the invaded and native range. Whether the dose or concentration of the allelochemical differs in the two ecosystems is not known.

In contrast to this criticism, the biogeographical approach utilized to study the invasive success of a non-native species is a significant and novel contribution to ecological patterning studies (Hierro et al. 2005). Callaway et al. (2005) found that some individuals of invaded populations were tolerant to *Centaurea* spp. invasion. Individuals grown from seeds of individuals that have survived allelopathic activities became resistant to *Centaurea* invasion, and co-existed successfully (Figure 1). The selection of resistant native species is thought to lead to

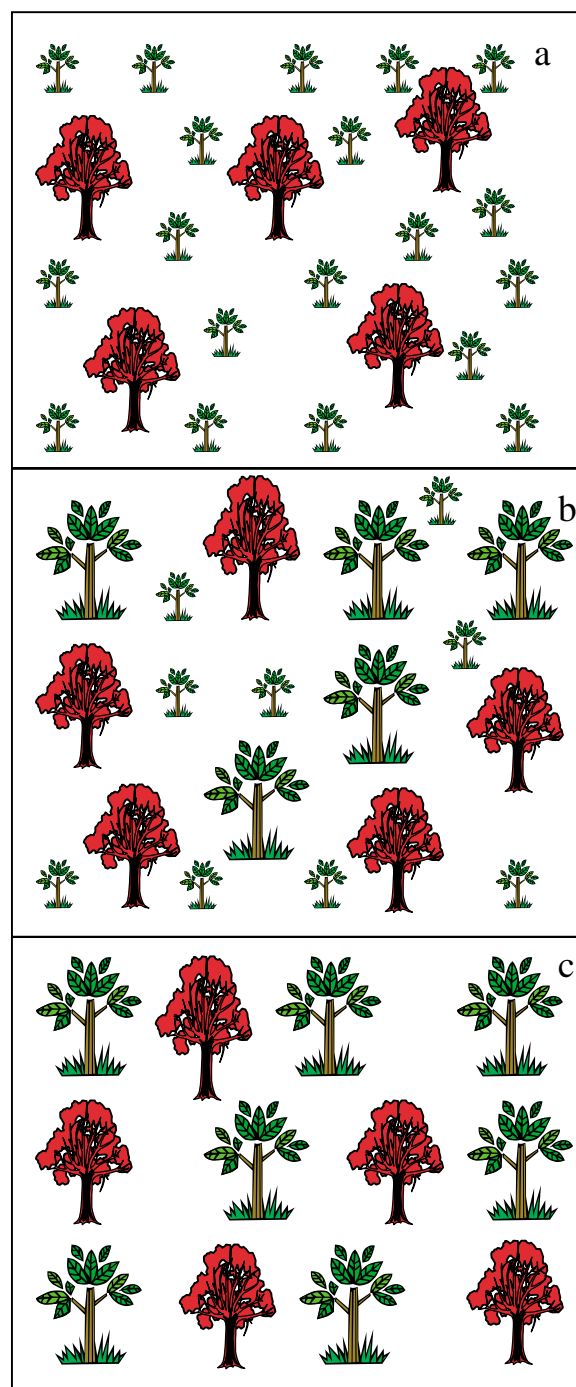


Figure 1. Schematic representation showing (a) growth suppression of native residents (shown in green color) following invasion of non-native (shown in red color) (Callaway & Aschehoug 2000); (b) individuals raised from seeds that survived invasion are resistant to phytotoxic activities of the invader (Callaway et al. 2005), and (c) selection of native residents that are resistant to invasion results in co-existence of non-native invasives and native residents.

coexistence of native residents and invaders. Native residents susceptible to knapweed allelochemicals will potentially be eliminated from the population over time in introduced ranges. This will result in the selection of native residents with resistance to the allelopathic activity of invaders, and coexistence of native residents and invasive non-native species over time, indicating a temporal effect associated with the

apparent initial success of allelopathy as an invasion strategy. Such a situation is largely similar to interactions now existing in the native range of *Centaurea*. It will be interesting to see how further research investigating plant interactions in this system evolves. That there are few examples of native species recovering significantly from invasions by highly competitive non-native plant species suggests that the situation described with regard to *Centaurea* spp. is rare.

Rice has been widely studied for its allelopathic potential (Olofsdotter 1998). Thousands of rice cultivars have been screened (e.g., Dilday et al. 2001, Kong et al. 2002), resulting in discovery of those that can suppress weeds such as barnyardgrass (*Echinochloa crus-galli*) (e.g., Dilday et al. 2001, Jensen et al. 2001) and *Cyperus difformis* (Kong et al. 2004a). Even though the level of weed management is not exceptional in comparison to that obtained by the application of synthetic herbicides; herbicide use rates can be substantially reduced by utilizing inherently weed suppressive rice cultivars (Chavez et al. 1999).

Allelochemicals reported from the root exudates of allelopathic rice varieties include momilactone B (Kato-Noguchi & Ino 2004), glucosides of two resorcinols, a glucoside of a flavone, glucosides of two benzoxazinoids (Kong et al. 2002) and a cyclohexenone (Kong et al. 2004a). Thus, more than one phytotoxin may play a role in the weed suppression observed in the most allelopathic varieties of rice. Most importantly, Kong et al. (2004b) found that the synthesis of two compounds that are phytotoxic to barnyardgrass, a flavone (5,7,4'-trihydroxy-3',5'-dimethoxyflavone) and a cyclohexenone (3-isopropyl-5-acetoxycyclohexene-2-one-1), are induced in rice plants by the presence of barnyardgrass or a particular compound produced by barnyardgrass. This is the first report of chemical induction of allelochemical synthesis by the presence of another plant species.

Genetic information related to the allelopathic potential of rice, such as quantitative trait loci mapping of allelopathic traits (Jensen et al. 2001), has been produced, but no direct link between this information to production of any particular allelochemical has been demonstrated. Identification of the genes involved in the production of the key allelochemicals in rice is needed in order to exploit the trait of bioactive root exudation. Availability of the complete rice genome suggests this will likely occur in the near future. Already, Xu et al. (2004) reported that the gene for rice *syn*-copalyl diphosphate synthase plays a regulatory role in the synthesis of the momilactones and structurally related phytoalexins. Blocking synthesis of one or more of these allelochemicals using iRNA methods could be useful in determining their potential roles in allelopathy.

Sorgoleone (2-hydroxy-5-methoxy-3-[(8'z,11'z)-8',11',14'-pentadecatriene]-*p*-benzoquinone) is a potential allelochemical that is exuded from the living root hairs of related sorghum species (Czarnota et al. 2001, 2003a, 2003b). From a weed suppressive standpoint, sorghum and its hybrids (*Sorghum* – sudangrass hybrid, *S. bicolor* × *S. sudanese*) are preferentially cultivated in United States as cover or green manure crops in many agronomic settings (Weston et al. 1999). A mixture of bioactive long-chain hydroquinones is released by living root hairs of *Sorghum bicolor* with sorgoleone generally being the major component (80–90%) of the mixture and the remainder being congeners and analogues of sorgoleone, often with similar biological activity (Czarnota et al. 2003a, Kagan et al. 2003, Rimando et al. 2003). Sorgoleone is a potent phytotoxin, causing growth inhibition in both broad leaf and grassy weed species grown in both hydroponic and soil assays at 10 µM concentrations (Nimbal et al. 1996, Czarnota et al. 2001). Oxygen availability and ethylene concentration at the root surface impact root hair development and sorgoleone production in a laboratory setting. By reducing O₂ and ethylene concentration on root surface, development of root hairs and resulting production of sorgoleone was completely inhibited (Yang et al. 2004a). Light also inhibits sorgoleone production while crude aqueous extracts of the weed *Abutilon theophrastii* were recently found to stimulate synthesis (Dayan 2006).

Sorgoleone apparently possesses multiple modes of action, but we do not know which of these modes is/are dominant in allelopathy. It affects chloroplastic, mitochondrial, and cell replication functions of higher plant cells. It binds the D1 protein coupled with electron transfer between Q_A and Q_B within photosystem II (PSII), thus inhibiting PSII (Gonzalez et al. 1997, Rimando et al. 1998, 2003). It inhibits respiration (Rasmussen et al. 1992) and root membrane H⁺-ATPase (Hejl & Koster 2004). It inhibits the activity of hydroxyphenylpyruvate dioxygenase (HPPD), thus reducing carotenoid biosynthesis and causing bleaching of sensitive tissues (Meazza et al. 2002). Multiple molecular targets of sorgoleone make it less likely that resistance to it will evolve in target species. *In vivo* activity of sorgoleone, however, differs from *in vitro* activity, likely due to its physicochemical properties or rapid degradation *in vivo*.

The biochemical pathway of sorgoleone has been tentatively characterized (Dayan et al. 2003), and genes encoding the enzymes for regulation and production of sorgoleone are now being identified (Yang et al. 2004b, Baerson et al. 2005b). Using differential display techniques with root hair producing and non-producing seedlings, a root hair-specific gene involved in sorgoleone biosynthesis was isolated (Yang et al. 2004a). More recently, other genes, involved in sorgoleone biosynthesis,

have been characterized by analysis of a root hair expressed sequence tag library (Baerson et al. 2005a). In this study, sorghum genes have been heterologously expressed and gene products have been found to have substrate specificities consistent with participation in the sorgoleone biosynthetic pathway. Plans are underway to transform *Sorghum* spp. with gene constructs that will alter expression of these genes in order to determine their function in sorgoleone synthesis. Phenotypic evaluation of transformants will be valuable in determination of the role of sorgoleone in allelopathic interactions. These results might have important implications with regard to enhancing the production of sorgoleone for additional weed suppression, and/or the introduction of these or related genes to crops lacking the ability to produce bioactive root exudates.

Although considerable progress has been made in several well-characterized systems to identify the allelochemicals involved in plant interference, locate sites of production, and determine mode(s) of action, further work remains to be done from an ecological perspective to clearly define the role of allelochemicals in such complex phenomena like plant interference, plant succession, and reduction in biodiversity of native species induced by exotic, invasive species in natural settings. Addressing these complex issues will require considerable creativity when considering plant-plant interactions, as well as knowledge of ecosystem and population ecology and soil chemistry, ecology and microbiology. This suggests that a large multidisciplinary approach may be needed to make significant progress in this area. One successful example of this type of approach in Europe involved chemists, physiologists and ecologists; they successfully investigated the ability of a winter rye cover crop (*Secale cereale*) to produce a variety of allelochemicals over time, which formed biologically active metabolites in various soil settings (Macias et al. 2005a, 2005b). This work was performed to determine the role of potential allelochemicals as phytotoxins or toxins in a managed agroecosystem. A similar multidisciplinary approach with access to substantial funding is needed to address the role of allelopathy and allelochemicals as phytotoxins in a natural or unmanaged environment.

Concluding remarks

In the last 10 years, allelopathy has progressed significantly as a science. Today, many more papers are published on this subject in high impact journals than in the past. Recent advances in our ability to isolate and identify minute quantities of biologically active substances, including allelochemicals, have led to our ability to study allelopathy in greater depth, especially in controlled environments. Moreover, the use of molecular tools to determine the genetic basis of allelopathy and to manipulate biosynthesis of

putative allelochemicals can provide evidence of the role of specific phytochemicals in allelopathic interactions. Although recent progress has been made in these areas, further developments are needed in evaluating the impact of allelochemicals upon soil macro- and micro-biota, soil properties, ecological patterning and succession. With the development of new molecular and chemical techniques to trace allelochemical production and persistence and to follow impacts of these compounds on species establishment, the role of allelopathy in plant-mediated interference in natural ecosystems is expected to become increasingly clear in the coming years. Only multidisciplinary efforts involving plant ecology, genetics, physiology, biochemistry, soil science, and microbiology, at a minimum, can address this complex research area and answer the key questions of if and how allelopathy influences plant interactions and invasive plant success over time.

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